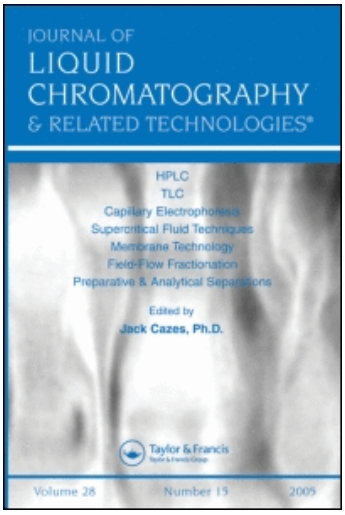


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# Analysis of Saturated and Unsaturated Phospholipids in Biological Fluids

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# Analysis of Saturated and Unsaturated Phospholipids in Biological Fluids

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## ABSTRACT

Cupric acetate (3% in 8% phosphoric acid) as a charring agent reacts only with unsaturated phospholipids while cupric sulfate (10% in 8% phosphoric acid) reacts with both saturated and unsaturated phospholipids. Thus, the amount of saturated phospholipid in a zone on a thin layer chromatogram (TLC) can be calculated by the difference in reactivity . An evaluation of methods shows that direct application of biological samples to TLC for separation and quantitation of phospholipids is reproducible. The use of these techniques for a number of different samples is described.

## INTRODUCTION

The literature has indicated that there was no universally accepted method for either separation or quantitation of phospholipids (PL) in biological fluids, particularly amniotic fluid. The problem was complicated by the fact that in spite of the availability of synthetic phospholipids, many investigators used natural sources without realizing that these are mixtures of saturated and unsaturated phospholipids. Thus, reports of quantitation of "dipalmitoyl" lecithin may in reality be erroneous since the reference material reportedly used was from natural sources. The methodology for determination of the individual phospholipids separated in a zone on a thin





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layer chromatogram is tedious, involving extensive detailed analysis (1-3).

The experiments described here present methodology to quantitate the unsaturated and saturated phospholipids separated on thin layer chromatograms. The traditional analysis of phospholipids in amniotic fluid has failed to consider that both the saturated and unsaturated homologues separate together on TLC. It has been reported that in living sperm there are varying amounts of saturated and unsaturated phospholipids (4-6). Gluck, et al., in their earlier reports indicated that as much as 30% of the lecithin in amniotic fluid is of the unsaturated homologue (7). Using the differentiation provided by reaction with cupric acetate and cupric sulfate (8) it has been possible to determine the ratio between these two classes in the phospholipids of anmiotic fluid and seminal fluid . The method also results in a value for the true"saturated" lecithin in various fluids. The determination of saturated lecithin in amniotic fluid has been shown by Torday et al., (9) to be a more reliable indication of lung maturity in perinates.

The cupric acetate reagent has been shown to react only with the unsaturated PL (8). The cupric sulfate reacts with both the saturated and unsaturated species. By interpolation on the respective standard curves and by difference the amounts of the two species can be determined.

Results of the use of this method for a number of biological samples are presented.

## MATERIALS

The synthetic phospholipids lysolecithin (LL), sphingomyelin (S), dipalmitoyl lecithin (L), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PEA), phosphatidylglycerol (PG), n-monomethyl phosphatidylethanolamine (PEN) and n-dimethyl phosphatidylethalomine (PENN) were purchased from Avanti Biochemicals, Birmingham, Alabama. These were dissolved in chloroform-methanol (1:1) at the concentration of 100 ng/μL. The purity of these reference substances was verified by chromatography in various mobile phases. For preparation of standard regression lines, 2-8 μL of standard were applied to the chromatoplate.

Whatman LK-5 layers (20 x 20 cm), 250 u thick, with preadsorbent zone, were scored on a Schoeffel scoring device to give 1 cm lanes. These were washed by continuous development overnight in chloroform-methanol (1:1). Development was carried out in standard size tanks. Copper (II) sulfate (anhydrous, Baker AR) was made as a 10% solution

in 8% phosphoric acid (8). Copper (II) acetate (monohydrate, Baker AR) was made as a 3% solution in 8% phosphoric acid (10).

The chromatograms were scanned in a Kontes Fiber Optic Scanner (Model 800) using a white phosphor disc. A Hewlett Packard model 3385A integrator provided the means of quantitation.

The scanning was carried out in the transmission mode using double beam operation. Scanning in the transmission mode gave higher results for the individual peaks than did scanning in the reflectance mode. This is in agreement with previous reports where transmittance was better than reflectance in scanning of substances separated on thin layers (11).

## METHODS for TLC and QUANTITATION

For evaluation of methodology presently in use for separation of PL from amniotic fluid the flow sheet in Figure 1 was followed. Labelled dipalmitoyl phosphatidyl choline was added to the sample and equilibrated at 4°overnight. Phosphatidyl choline, L-α dipalmitoyl-1 -$^{14}$C ) (0.01 mCi) 1000 m Ci/m Mol was obtained from New England Nuclear. This was dissolved in toluene-methanol (1:1) to give a solution of 2.22 x $10^7$dpm in 5 ml. Aliquots of this were taken for the determination of recovery at each step of the flow sheet. For 2.5 ml of amniotic fluid 25 µl of this stock solution was used. Recovery was based on counts in aliquots taken from the extracts in each step.

Amniotic fluids were analyzed as soon as received or kept frozen. Samples were not allowed to remain at room temperature but kept in ice for transport to the laboratory. They should be immediately frozen otherwise the phospholipid levels diminish. Seminal fluids were analyzed within the hour of collection. The samples (both amniotic fluid and seminal samples) were not centrifuged but applied directly to the preadsorbent zone of the LK5 layers. The application of untreated amniotic fluid was performed using a 25 ul microcapillary. Seminal fluids were diluted 1:1 with water and 10 ul was applied to the layer. During application a warm air blast from a hair dryer facilitated evaporation. The samples were applied across the lanes within the middle third of the application area with drying between each application of amniotic fluid until 50 ul (last month) or 100 ul (earlier gestation) volumes of amniotic fluid had been applied. Standards for reference are applied on other lanes and only alternate lanes are used. The samples were applied in duplicate, one each on different halves of the 20 x 20 plate. When many samples were to be analyzed, duplicates were applied to each of two whole plates.

After drying, the layers are predeveloped twice with 1:1 chloroform-methanol to the interface of the preadsorbent zone. This serves to extract the phospholipid from the sample and deposit it as a line on the starting point of the chromatogram. The developing solvent was chloroform-ethanol-triethylamine-water (30:30:34:8) (8). Development was allowed to proceed until the mobile phase front had reached 2 cm. from the top of the plate. This usually required 1-1.5 hours.

In the mobile phase described, cardiolipin (diphosphatidyl glycerol) migrates near phosphatidyl glycerol ($R_f$0.70 vs. $R_f$0.64). In order to verify the lack of cardiolipin in amniotic fluid, aliquots of the same sample were subjected to TLC on Analtech H plates in the mobile phase, chloroform-methanol-acetic acid-water (60:14:13)2) (12). In this system, PG shows $R_f$ of 0.60 vs. that of cardiolipin of 0.85. Eight amniotic fluids showed no detectable cardiolipin, however, all of the seminal fluids contained this substance.

After development, the chromatogram was dried in air, then in an oven at 170$^{O}$ for 2 min. to remove residual solvent. The plate was cut in half with a glass cutter, or two 20 x 20 cm plates are used if multiple samples were separated. One half was sprayed with the cupric acetate reagent and the other half sprayed with the cupric sulfate reagent. The chromatograms were sprayed until throughly wet. The cupric acetate plate is allowed to dry in air for 5 min., heated at 110$^{O}$ for 5 min., then heated in an oven at 180$^{O}$for 10 min. The cupric sulfate plate is allowed to dry in air for 5 min., heated in an oven for 5 min. at 120$^{O}$C, then heater at 170$^{O}$ C for 10 min.

The chromatograms are then scanned by densitometry. They should be scanned within the hour or stored in the dark until scanned.

From the standard curve set up by the scanning of serial amounts (2.0 - 8.0 μg) of phospholipid, the amounts of the individual phospholipid can be interpolated. The amount of the " unsaturated" phospholipid is obtained from the equivalent integrated curve (cupric acetate spray).

The amount of the "saturated" phospholipid is determined by use of the cupric sulfate curve followed by subtraction of the amount of the unsaturated phospholipid since the cupric sulfate reacts with both. Actual amounts can then be plotted against gestational age to give a working curve in the case of amniotic fluids.

## RESULTS and DISCUSSION

Early in assessment of the assay of phospholipids in amniotic fluids as described by Gluck (and the many variations), it became

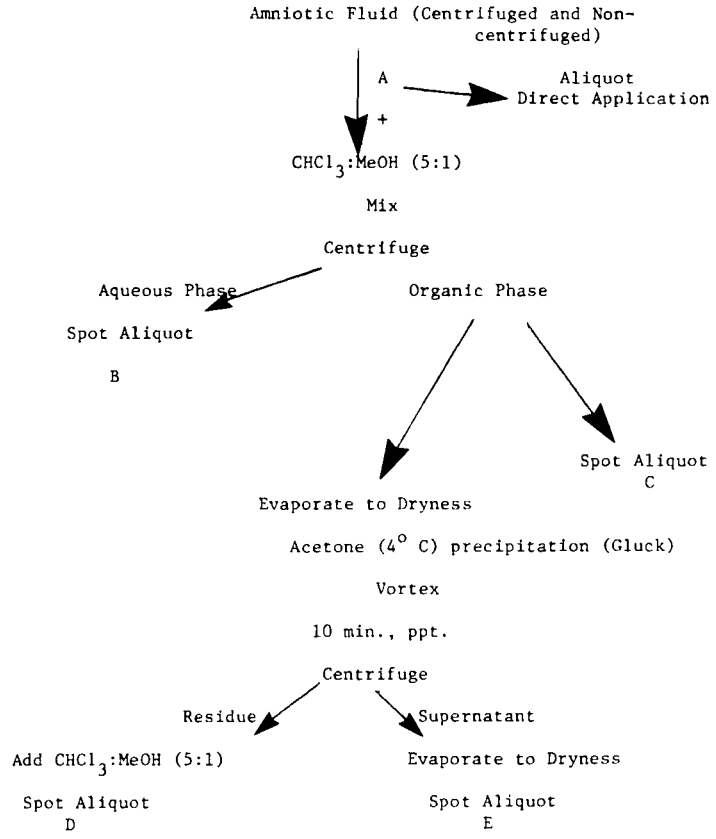


Figure I Scheme for Evaluation of Extract Procedures

apparent that the quantitative aspects of PL determination have been neglected. Using DPL-$^{14}$C (see methods) a study of the distribution of DPL-$^{14}$C in the various fractions of the procedure (see Fig. 1) presently in use was performed. As much as 35% of added radioactivity was lost to the precipitate (A) from the amniotic fluid when centrifugation was included in the first step. The supernatant contained 65% of the counts. These results are in agreement with those of Oulton (13). The extraction of the phospholipid form the amniotic fluid by chloroform-methanol (5:1) (D) was quantitative. The distribution of the radio-activity between the supernatant and the precipitate as the result of the acetone precipitation was variable. There was from 6-28% of the radioactivity in the supernatant (E). This result may be due to the difficulties related to the reproducible removal fo the acetone after

TABLE I

Reactivity of Phospholipids Toward Charring Reagents

| | | Character [a] | Acetate [b] | Sulfate [c] |
|---|---|---|---|---|
| 1. | Dimyristoyl PC[d] | S | - | + |
| 2. | Dipalmitoyl PC | S | - | + |
| 3. | Distearoyl PC | S | - | + |
| 4. | 1-Palmitoyl-2-oleoyl PC | U | + | + |
| 5. | Dioleoyl PC | U | + | + |
| 6. | Dilinolenyl PC | U | + | + |
| 7. | Dilinoleoyl PC | U | + | + |
| 8. | Lecithin (beef heart) | mix | + | + |
| 9. | Phosphatidyl ethanolamine | mix | + | + |
| 10. | Phosphatidyl serine (bovine brain) | mix | + | + |
| 11. | Phosphatidyl inositol (bovine heart) | mix | + | + |

a. Saturated (s) or unsaturated (u)
b. Reaction with cupric acetate as described
c. Reaction with cupric sulfate
d. Phosphatidyl choline

centrifugation as well as possible evaporation of the acetone although the sample was kept at $0^{\circ}$throughout the procedure. The precipitate (F) thus contained variable amounts of the PL. Table 1 shows the results obtained when various synthetic phospholipids were subjected to charring with the two reagents. Unsaturated phospholipids reacted with both the cupric acetate and cupric sulfate reagents. The saturated PL reacted only with the copper sulfate reagents. Also indicated is the presence of both saturated and unsaturated material in purified material from natural sources. Manufacturers literature usually show that the supplied material is not a single entity.

Evaluation of the amounts of "saturated" versus "unsaturated" lecithin after chromatographic separation and differentiation with the cupric acetate and cupric sulfate sprays showed that 33% of the lecithin in (F) was "unsaturated" and the remainder was present as "saturated" lecithin. With different amniotic fluids the proportions were different. Gluck et al., (7) reported that there were both unsaturated as well as saturated lecithins in the acetone precipitate. The lecithins in the supernatant (E) of the acetone precipitation step showed both saturated and unsaturated species. Since the classical acetone precipitation procedure for separation of the saturated from unsaturated phospholipid called for cooling to $-60^{\circ}$C and $-20^{\circ}$ C, there appears to be no basis for use of the acetone precipitation step of the various methods, unless there is present in the lecithin fraction of the chromatograms a substance which can account for the surface activity present in excess of that expected from the phospholipid.

Recovery of Lecithin from Amniotic Fluid and from the Sorbent of the TLC.

Evaluation of the TLC was performed by determination of recovery of the DPL-$^{14}$C (see methods) added to 1) amniotic fluid before aliquot applications, 2) to amniotic fluid after application and 3) directly to the preadsorbent layer. The results showed that the preadsorbent layer retained little if any of the lecithin; the recovery of radioactivity from the lecithin area of chromatogram was over 95% in each of the three instances. Less than 0.3% of the added radioactivity was recovered from the preadsorbent area of the chromatogram.

Quantitative Aspects of Densitometry of TLC of PL.
Reproducibility of use of the charring reagents as described in Methods evolved from the finding that heating of the TLC plate in a convention oven was erratic. This is a major problem since time and temperature are critical to reproducibility. Furthermore, it is difficult to reproduce the conditions of spraying from plate to plate. The problem was solved by pre-heating the chromatograms at $110^{\circ}$ C for 5 min. in one oven to remove the water of the spray before the charring in a second

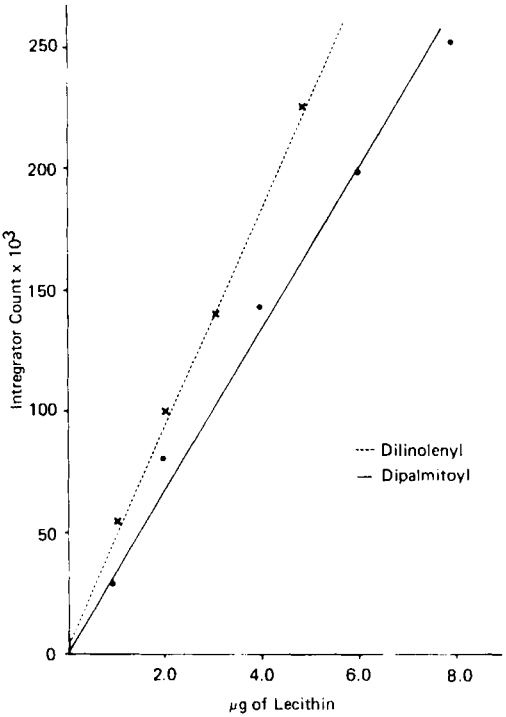


Figure II Standard Curve for Saturated and Unsaturated Lecithins
For Lecithin Curve : y=29.214x-0.375
(n-28) R-0.984



oven at the higher temperature. The latter oven was modified to include a metal baffle between the door and the oven in order to prevent heat loss when the door was opened. It contained a slit , the dimensions of which permitted a 20 x 20 cm. thin layer plate to be inserted in the oven without a gross drop in temperature. This improvement of technique resulted in reproducibility of quantitation of the charring of the phospholipids and other materials.

Figure 2 shows the calibration curves for dipalmitoyl lecithin and dilinolenyl lecithin. The slopes of these curves are reproducible. (See legend of the figure for evaluation of linear regression). The curve for the first was obtained using cupric sulfate as reagent. The curpic acetate has never been found to react with dipalmitoyl lecithin or other saturated PL. If a reaction is seen with the cupric acetate reagent it must be concluded that an unsaturated PL is present.

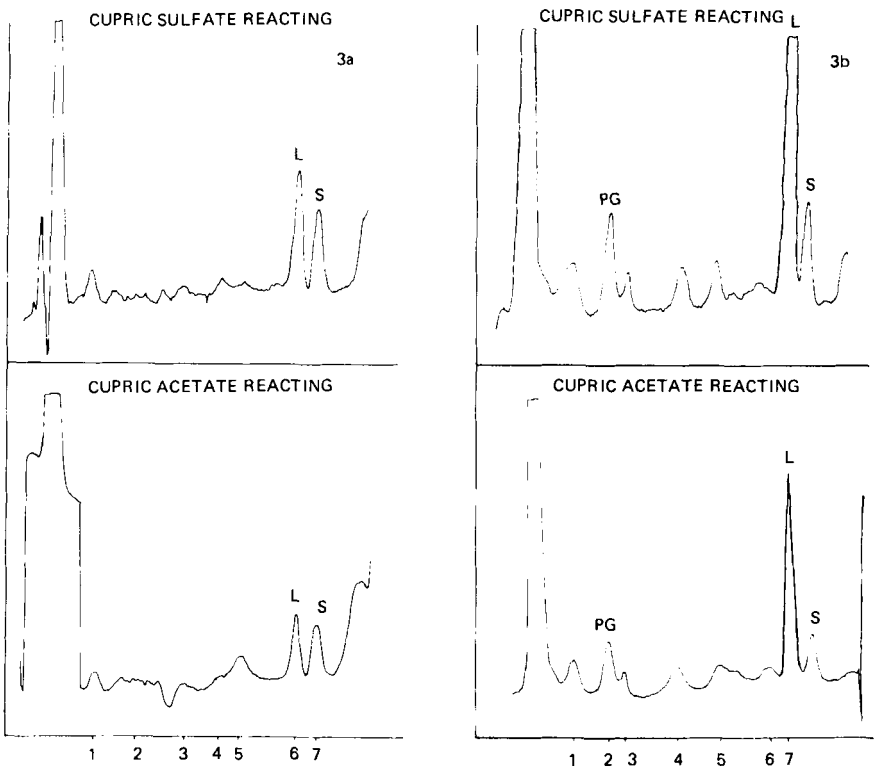


Figure III Scans of chromatogram of amniotic fluid of early pregnancy a) and late pregnancy b) after cupric acetate and cupric sulfate detection.

Replicate samples of amniotic fluid were applied to a number of plates and reproducibility of the method determined from day to day and within the day for the quantitation of lecithin. Day to day (n=80) reproducibility gave a coefficient (CV) of variation of 9.7%, within day (n=45) the CV was 6%, within a plate (n=9) CV was 3.6%. These results were obtained with amniotic fluids carried through the entire procedure including scanning on the densitometer. It was found that without the pre-washing of the plates the backgrounds after charring were not reproducible, varied widely, and gave erratic results even in within day experiments.

Figure 3 shows composite scans of the chromatograms obtained after charring with cupric acetate and with cupric sulfate reagents for amniotic fluids from early (A) and (B) late pregnancy. It appears that

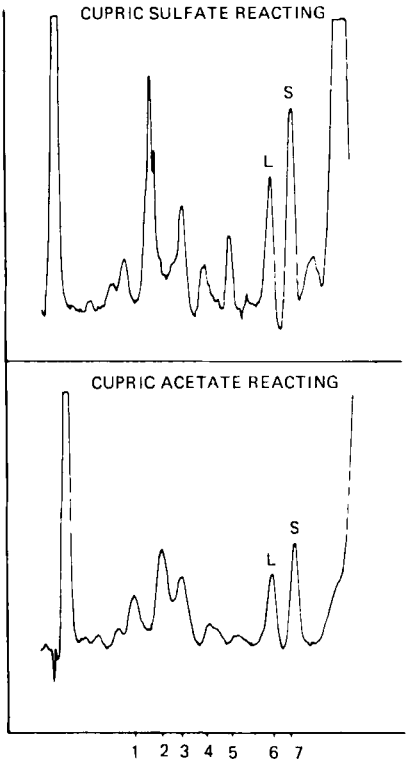


Figure IV Scans of chromatograms of seminal fluid after cupric acetate and cupric sulfate reactions.



in early stages of pregancy little PL other than L and S are present in appreciable amounts. Figure 4 shows the scans from a sample of seminal fluid carried through the same procedure. There is a larger proportion of sphingomyelin in these samples and more recent evidence indicates that the greater proportion of spingomyelin is found in the seminal plasma, while other PL are derived from the sperm. By correlating the integrated areas of these scans, information can be obtained regarding the relative amounts of percent unsaturated and saturated species in each of the separated phospholipid areas of the chromatograms.

Figure 5 shows the results of correlating the amount of "disaturated" lecithin found in 60 amniotic fluids with the gestational age. As indicated in this figure, the "disaturated" lecithin shows a sharp increase in concentration in the period after 34 weeks.

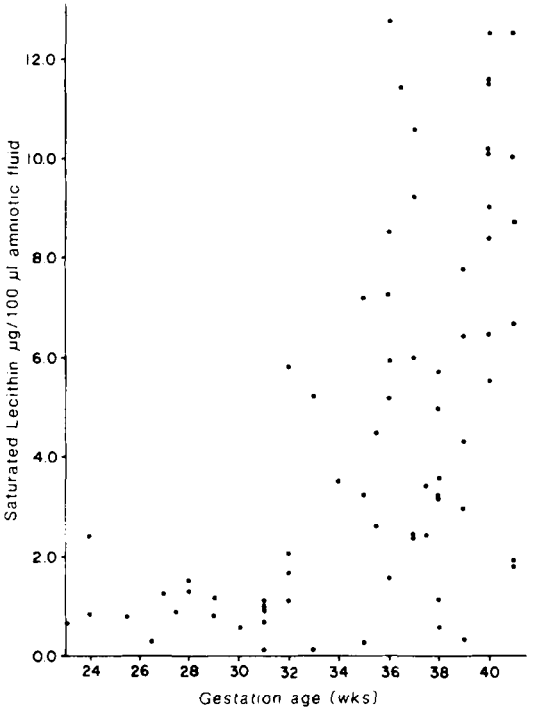


Figure V Scattergram resulting from correlating gestational age with amount of "disaturated" lecithin



A differentiation was made of the amounts of unsaturated and saturated PL in various commercially available samples and some biological tissues. Table II shows the amounts found in these sources. The relative amounts of saturated verses the unsaturated lipids varied widely depending on the source. The nature of the constituents in the saturated as well as the unsaturated fractions are unknown. It has been shown in earlier work on amniotic fluid by Gluck et al., (7) and by Selivonchick et al., (2) that the lecithin fraction contains both saturated and unsaturated compounds. The method described gives a reproducible, simple means of separating the phospholipids and determining the amount of unsaturation of each.

There has been some controversy over the validity of the use of cold acetone precipitation in methodology of phospholipid determination. A number of studies have shown that the acetone precipitated lecithins

TABLE II

Phospholipids in Biological Samples

Amount in Applied Sample (ng)

| Source | Lecithin S[a] | Lecithin U[b] | Sphingomyelin S | Sphingomyelin U | Phosphatidylglycerol S | Phosphatidylglycerol U |
|---|---|---|---|---|---|---|
| Egg Lecithin | 120 | 40 | ND[c] | ND | ND | -- |
| Bovine Heart Plasmalogen | 275 | 150 | ND | -- | ND | -- |
| Mouse Serum 10 ul | 3950 | 800 | 280 | 105 | ND | -- |
| Seminal Fluid Human, 5 ul | | | | | | |
| 1. | 700 | 150 | 63 | 630 | ND | -- |
| 2. | 350 | 100 | 43 | 187 | ND | -- |
| 3. | 350 | 100 | ND | 402 | ND | -- |
| 4. | 1150 | 250 | ND | 1200 | | |
| Amniotic Fluid 100 ul | | | | | | |
| 1. 23 weeks | 670 | 270 | ND | 600 | ND | -- |
| 2. 32 weeks | 1680 | 720 | ND | 865 | ND | -- |
| 3. 35 weeks | 3220 | 780 | ND | 585 | ND | -- |
| 4. 38 weeks | 3850 | 1800 | ND | 335 | ND | -- |
| 5. 40 weeks | 9750 | 3750 | ND | 140 | 1650 | 690 |
| 6. 40 weeks | 6900 | 2630 | ND | 950 | 1250 | 725 |
| 7. 42 weeks | 5520 | 2480 | ND | 230 | 1250 | 720 |

a S = saturated b U= unsaturated c ND= not detected

are not solely composed of saturated molecules (14, 15). In our earlier work, we found saturated as well as unsaturated phospholipids in the acetone precipitate as well as the supernatant. This is in agreement with Torday et al., (9). There also has been disagreement as to the advisability of centrifuging the amniotic fluid prior to extraction. The fact that amniotic fluids contain different concentrations of protein in various samples may in a way explain why there is a variation in the results from different laboratories. Centrifugation of the amniotic at different speeds as shown here results in loss of phospholipids. Different concentrations of protein ("debris") will only serve to magnify the variability of error if it is considered that protein binding of the phospholipid is the factor. For this reason, centrifugation was omitted form the present mehtod for both the amniotic and seminal fluids.

The method described is reproducible as illustrated by the calibration curve. However, it is recommended to use internal or external standards for each chromatogram to compensate for normal analytical variability. This practice should be observed in any analytical chromatographic procedures. It has been shown in each step of the procedure that quantitative evaluation and real, reproducible data result. The procedure may serve to provide further data in phospholipid metabolism.



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